Plant Cell-Wall Degradation and Glycanase Activity of the Rumen Anaerobic Fungus *Neocallimastix frontalis* MCH3 Grown on Various Forages

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ABSTRACT : Studies were made of digestion of timothy *(Phleum pratense)* hay, tall fescue *(Festuca elatior)* hay, and rice *(Oryza sativa)* straw in pure cultures of rumen anaerobic fungus, *Neocallimastix frontails* MCH3. The fungus was inoculated on ground forages (1%, w/v) in an anaerobic medium and incubated at 39℃. Incubation was continued for 24, 48, 72 and 96 h. The losses of dry matter, xylose and glucose of forage during incubation were determined at the end of these incubation periods. Xylose and glucose were considered to be released from xylan and cellulose, respectively. The digested xylan to digested cellulose (X/C) ratios of the substrate were calculated. Xylanase and carboxymethyl cellulase (CMCase) of culture supernatant and residual substrate was measured at the same time. The X/C ratios in the cultures on timothy hay and rice straw were greater than 0.5 in the first 24-h incubation period. The values were smaller than 0.3 in tall fesque. The ratio of xylanase activity to that of CMCase in the first 24-h incubation period correlated well with the traits in X/C ratio. However xylanase activity was still superior to CMCase in the following incubation period (48 to 96 h), although the glucose (designated as cellulose) was more intensively digested than xylose (designated as xylan). The production of these polysaccharidases appeared to correlate with substrate cell-wall sugar composition, xylose to glucose ratios, at the beginning of fast growing period. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 5 : 752-757*)

Key Words : Anaerobic Rumen Fungi, Fiber Degradation, Cellulase, Xylanase

INTRODUCTION

Fiber-digesting rumen microbes are usually characterized by their preference for hemicellulose (xylan), cellulose or pectin (Hungate, 1966). Obviously "cellulolytic" organisms prefer using cellulose to xylan and vice versa for "hemicellulolytic" organisms (Dehority and Scott, 1967; Coen and Dehority, 1970). The traits in fiber degradation of particular organisms have been successfully characterized by digested xylan to digested cellulose (X/C) ratio during pure culture on timothy hay (Matsui et al., 1998).

A cellulolytic bacterium, *Fibrobacter succinogenes* S85, showed a low X/C ratio (<0.3) and a hemicellulolytic bacterium, *Prevotella ruminicola* subsp. *brevis* 7-31, showed consistently higher X/C ratio (>0.5) throughout incubation. However, a rumen chytridiomycete 'fungus *Neocallimastix frontalis* MCH3 was different from these bacteria in showing a particular trait in X/C ratio; this fungus showed high X/C ratio (>1.0) at the beginning of incubation, then its X/C ratio declined to as low as that of *F. succinogenes*. Therefore this fungus appeared to degrade xylan first and preferentially degrade cellulose later. This seems to be reasonable for organisms that possess cellulase as well as xylanase activities, because cellulose microfibrils are surrounded by hemicellulose in the plant cell wall (Fry, 1986). Therefore *N. frontailis* MCH3 was "xylanolytic" at the first stage of fiber degradation, then tended to be "cellulolytic" later. We did not know, however, if this trend is always true for this fungus, because the trend in X/C ratio may be affected by chemical composition of plant materials degraded. Moreover the relation between particular polysaccharidase production and their substrate degradation is yet to be determined in this context, because *Neocallimastix spp.* have been reported to produce more xylanase than cellulase irrespective of growth substrate according to the profiles of glucanase production (Lowe et al. 1987a; Williams and Orpin, 1987; Matsui et al. 1992; Teunissen et al. 1993). Indeed, there were no published results available about the relationship between enzyme production and the degradation of specific substrates by particular polysaccharidases in the rumen fibrolytic microorganisums. In this experiment we examined if the polysaccharidase production is related to the substrate plant cell-wall sugar composition and X/C ratio in the culture of *N. frontalis*.

MATERIALS AND METHODS

Organisms and culture

Anaerobic rumen fungus *N. frontalis* MCH3 was used in this experiment. This fungus was isolated from sheep rumen and kindly donated by Dr. G. Fonty (Laboratoire Microbiologie, INRA, Centre de Clermont-Ferrand-Theix, France). The fungus was routinely maintained according to Joblin (1981) in a medium containing timothy hay. Antibiotic, agar and cellobiose

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were omitted from the original medium in this experiment. Timothy (*Phleum pratense*) hay, rice (*Oryza sativa*) straw and tall fesque (*Festuca elatior*) hay were used in this experiment. The former two forages were commercially available products and the latter was obtained from Dr. H. Kawamoto (National Grassland Research Institute, Nishinasuno, Tochigi, Japan). These forages were ground to pass through 1-mm sieve by Wiley-mill. The forage particles (1% w/v) were added to 10-ml Joblin's medium as the sole energy source. Culture of fungus was done anaerobically (under 100% CO_2) and aseptically in Hungate-type glass tubes with butyl rubber septa (Bellco Glass Ware Inc, Vineland, NJ, USA). After at least three transfers on each substrate, fungus was inoculated to the test media and incubated for 24, 48, 72, and 96 h with occasional gentle shaking. Five tubes were allotted to each incubation period. Three of them were used for sugar analysis and the remainder for enzyme analysis.

Detergent analysis on substrate forages and monosaccharide analyses on residual substrates

Forages were analyzed for neutral and acid detergent fiber (NDF and ADF) contents. Klason lignin (acid detergent lignin, ADL) was also determined on acid detergent residues. NDF, ADF and ADL were analyzed according to Van Soest (1983). Acid hydrolysis and determination of neutral sugar as their alditol acetates by gas-chromatography was carried out according to Ushida et al. (1990) with slight modifications. After incubation was completed, culture tubes were centrifuged at 2,500×g for 15 min at 4℃ and supernatants were removed. Pellets from three tubes were exhaustively washed with distilled water and dried to constant weight at 80℃. Following the dry matter (DM) determination, a portion (10 mg) of dried pellets was ground with 0.4 ml 12 M H_2SO_4 in a 20-ml plastic tube by means of MINI-beads beater (Biospec Products, Inc., Bartlesville, Okla., USA) for 5 min at maximum speed. These homogenized forage pellets were hydrolyzed by H_2SO_4 at ambient temperature for 60 min, then transferred to glass test tubes with Teflon lining plastic caps. Distilled water was dispensed into the tubes to obtain 1 M H_2SO_4 and heated at 121℃ for 20 min under nitrogen atmosphere. After neutralization with aqueous ammonia solution (28%), free sugars were derivatized to alditol acetate and quantified by gas chromatography. Xylose and glucose disappearances from the substrates were respectively regarded as xylan and cellulose degradation in this experiment. The digested xylan to digested cellulose (X/C) ratio was calculated for every 24-h incubation period according to Matsui et al. (1998). Free neutral sugar in culture supernatants were also determined by the same gas chromatography technique.

Enzyme assay

Extraction of enzyme from fungi was done principally by the method of Williams and Orpin (1987). Briefly, pellets from two tubes were washed three times with 5 ml MES (2-morpholinoethanesulonic acid) buffer (25 mM, pH 6.5) for one g of pellet and sonicated for 5×60 s in a crushed ice bath (50W, TOMY UD-201, TOMY Co. Ltd., Tokyo, Japan). The supernatants after centrifugation at 20,000×g for 15 min at 4℃ were stored at -20℃ until assayed for endoglucanase (carboxymethycellulase; CMCase), and xylanase. Culture supernatants were also stored at -20℃ until assayed for same enzyme. The former fraction was designated as cell-bound enzyme and the latter as extracellular enzyme. CMCase and xylanase activities were assayed according to Flint et al. (1991) using CMC (Sigma) and Oat spelt xylan (Aldrich). Protein concentrations were determined by dye-binding method, but were not reported, because plant materials remaining in the samples appeared to significantly disturb the determination. Enzyme activity was therefore expressed as μmole glucose or xylose released/min/tube.

Chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) and Nacalaitesque (Kyoto, Japan) unless otherwise stated.

RESULTS

NDF, ADF and lignin contents (%) of timothy hay, tall fescue hay, and rice straw are shown in table 1. Timothy and tall fescue hays had relatively similar NDF and ADF contents. Rice straw had consistently higher fiber contents than two other substrates. Lignin content was substantially higher in rice straw (6.5%) followed by timothy hay 3.3%. Tall fescue hay had the lowest lignin content (2.0%).

Table 1. Fibre contents of forage substrates (% of dry matter)

	Timothy hay	Tall fescue hay	Rice straw
NDF	64.8±0.80	61.0±1.88	74.6±2.46
ADF	32.7±1.45	33.0±1.70	41.4±0.13
ADL	3.3±0.50	2.0±0.12	6.5±1.94

Values are means of three determinations with their standard deviations.
NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin.

The rates of DM degradation are shown in figure 1-a. The fungus MCH3 degraded timothy hay by ca. 50%, and tall fescue hay and rice straw by ca. 35% in 96h. DM degradation (figure 1-a) followed diauxic curves.

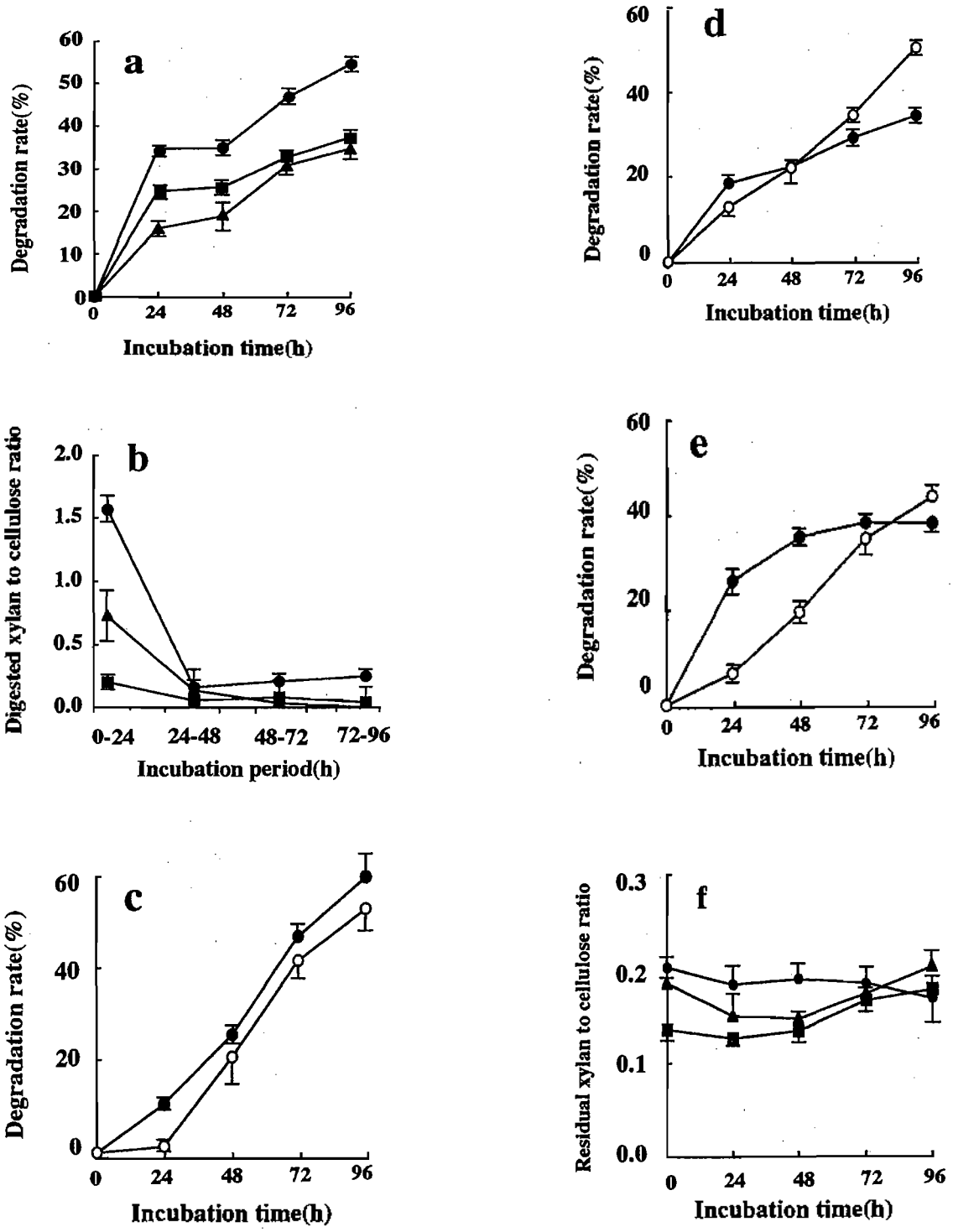

Figure 1. Substrate degradation by *Neocallimastix frontalis* MCH3

a. Dry matter degradation (%); ●, Timothy hay; ■, Tall fescue hay; ▲, Rice straw

b, Digested xylan to digested cellulose (X/C) ratio. ●, Timothy hay; ■, Tall fescue hay; ▲, Rice straw

c, Xylan and cellulose disappearance (%) from timothy hay. ●, xylose; ○, glucose

d, Xylan and cellulose disappearance (%) from tall fescue hay. ●, xylose; ○,glucose

e, Xylan and cellulose disappearance (%) from rice straw. ●, xylose; ○, glucose

f, Residual xylan to residual cellulose ratio. ●, Timothy hay; ■, Tall fescue hay; ▲, Rice straw

Values are means of two determinations. Bars represent standard deviations.

This may reflect the fungal growth pattern. Ruminal fungi have two life stages: a motile zoospore-stage and an inmotile vegetative stage. As the former makes little contribution to the plant cell-wall degradation, only the latter form of fungi degrades plant cell-wall. The life-cycle of rumen fungi is completed in about 24-32 h (Lowe et al., 1987b), the fast growing period of the first generation occurred during 0-24 h incubation. Maturation of sporangia followed by zoospore-release occurs thereafter and the fast growing period of the second generation appears to occur after 48-72 h incubation.

The xylose disappearance rate (regarded as xylan degradation rate) and the glucose disappearance rate (regarded as cellulose degradation rate) are shown in figures 1-c,d,e respectively for timothy hay, tall fescue hay and rice straw. Xylan degradation rates were higher in the first 24h than cellulose in timothy and rice straw cultures and this trait was reflected in the high X/C ratio (>0.5) of these forages in that period. This agrees with our previous observation (Matsui et al., 1998). On the contrary, xylan was degraded at the similar rate as cellulose in tall fesque cultures during the first 24 h incubation period. Its X/C ratio (0.2) was therefore smaller than that of timothy and rice straw in this period. However, the rate of cellulose degradation in timothy and rice straw increased in the following incubation period (24-48 h) and the X/C ratio of these substrates decreased to less than 0.5. The ratios of xylan to cellulose of the residual substrates at 0, 24, 48, 72 and 96 h incubation are shown in figure 1-f and the ratios were relatively constant.

CMCase and xylanase activities in both cell-bound and extracellular fractions are shown in figure 2. Although the cell-bound and extracellular enzyme activities increased with time in most cases, the magnitudes of increment were different between xylanase and CMCase. Indeed, the ratios of total xylanase activity to that of CMCase (figure 2-d) were not uniform for the forage species and the particular incubation period. The ratio of xylanase to CMCase activities in tall fescue culture was smaller than 1.0 during the first 48 h incubation period, while the values were larger than 1.0 in the case of timothy and rice straw. These ratios of enzyme activity at 24 h showed a similar tendency to the X/C ratio; the ratios of enzyme activity in tall fescue, rice straw and timothy cultures were placed in the same increasing order as observed in X/C ratio.

DISCUSSION

Morris and Bacon (1977) found that the monosaccharide composition of plant cell-walls affected the digestibility of forage in the rumen. The degradability of the plant cell-wall is also influenced by lignification (Buxton and Casler, 1993; Chesson and Forsberg, 1988). These factors affect degradability of hemicellulose, and accessibility to cellulose. These factors may also affect enzyme production (or induction) of the fibrolytic organisms. Although there were small differences in NDF and ADF contents between timothy and tall fescue hay, the proportion of hemicellulose (NDF-ADF) to cellulose (ADF-ADL) in tall fesque was lower than in timothy (28.0/31.0 *vs.* 32.1/29.4). That cell-wall sugar contents also suggested a relatively low proportion of xylan to cellulose in tall fescue; 0.13, and 0.20 respectively for tall fescue, and timothy (figure 1-f). The low proportion of xylan appeared to induce less intensive xylanase production and hence lower ratio of xylanase to CMCase activity in tall fescue culture. Fine structures of polysaccharides including the crystallinity of cellulose have been identified as one of the determinants in cell-wall degradation (Chesson and Forsberg, 1988).

Cellulose digestion of tall fescue hay required a shorter lag time (figure 1-d) than the other two forages (figure 1-c, e) suggesting easier access to cellulose or the easier degradation of cellulose molecules in this hay than the other two forages due to the relative low xylan to cellulose ratio. The tall fescue hay used here was prepared at the heading stage, while timothy hay was prepared at the late blooming stage (Matsui et al., 1998). The age of plants apparently affects fine structures of cell-walls (Lam et al., 1990) and might have led to relatively higher cellulase production in tall fescue cultures than in the other two forages in this experiment. Lignin content seems not to be closely related to enzyme production by this fungus because tall fesque hay had similar lignin content to timothy hay. There have been reports that lignin does not have an inhibitory effect on cellulase and xylanase production in *Neocallimastix* and *Aspergillus* (Stewart et al., 1983; Lowe et al., 1987a).

In spite of the good correlation between enzyme profile (Xylanase to CMCase) and trait in cell-wall polysaccharide degradation (X/C) during the first 24 h culture, they did not correlate well in the following culture periods. The significant reduction in X/C ratio indicating a relative decrease in xylan degradation to cellulose contrasted with the relatively high xylanase to CMCase ration maintained in timothy and rice straw cultures. The exact reason for this is still unknown. One possibile explanation is the residual xylan to residual cellulose ratio of substrates. The proportions of xylan and cellulose at 48 h when the second fast growth period started were still largely maintained (figure 1-f). Such relatively unchanged proportions of two major cell-wall sugars led to similar enzyme production, in the second fast growing period.

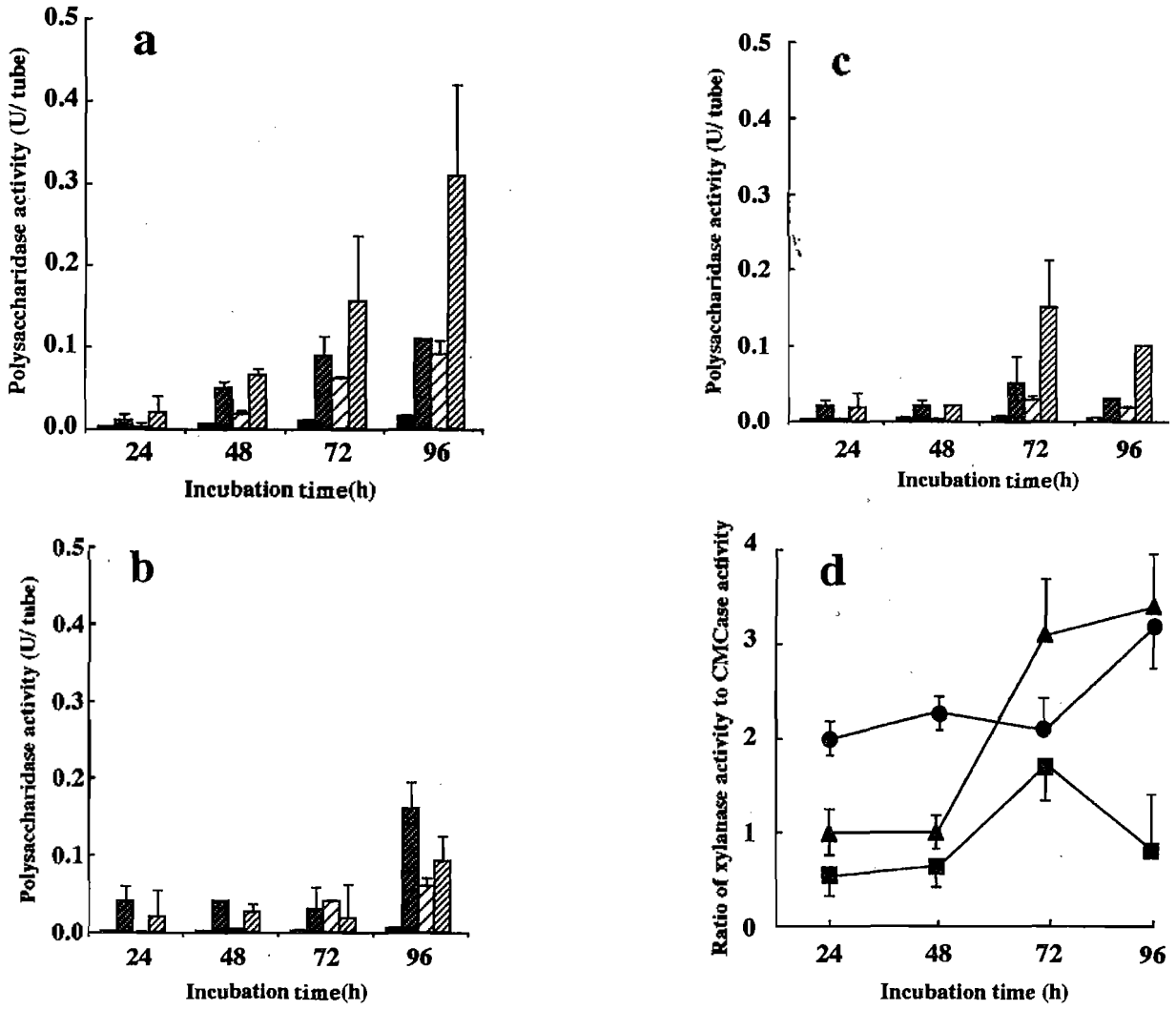

Figure 2. Endoglucanase (CMCase) and xylanase activity in fungal culture
a. Timothy hay culture; b, Tall fescue hay culture; c, Rice straw culture, d, Ratio of xylanase activity to CMCase activity.
a,b,c : ■, Cell-bound CMCase; ▩, Extracellular CMCase; ▨, Cell-bound xylanase; ▨, Extracellular xylanase.
Unit= μ mol glucose or xylose released from carboxymethylcellulose or oat spelt xylan/min. Details see text.
d: ●, Timothy hay culture; ■, Tall fesque hay culture; ▲, Rice straw culture.
Values are means of two determinations. Bars represents standard deviations.

CONCLUSION

CMCase and xylanase production by *N. frontalis* MCH3 was modulated according to the cell-wall sugar composition. This was the most evident in the early incubation period. If intensive xylan removal is required to attack cellulose, this fungus appeared to achieve it efficiently. Since this fungus is able to use a wide variety of carbohydrate sources as growth substrates (Bernalier, 1991), oligo- and mono-saccarides produced by polysaccharidase action are well consumed. Indeed, small amount of xylose (8 to 20 μ g/tube) and glucose (30 to 90 μ g/tube) were detected in the culture supernatant at 96 h. This was distinct from major rumen cellulolytic bacteria as *F. succinogenes* and *Ruminococci*. These bacteria cannot use xylose as the growth substrate even though they produce xylanase (Hungate, 1966). This represents an important advantage for this fungus in the rumen environment.

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REFERENCES

Bernalier A. 1991. Les champinions anaerobies du rumen. Ph.D. thesis. Universite Blaise Pascal-Clermont-Ferrand II.N. DU 281. 182pp.

Buxton, D. R. and M. D. Casler. 1993. Environmental and Genetic Effects on Cell Wall Composition and Digestibility. In: Jung, H. G., Buxton, D. R., Hatfield, R. D., and Ralph, J. (Ed). Forage Cell Wall Structure and Digestibility. Madison, USA:ASA, CSS and SSA. p. 685-714.

Chesson A. and C. W. Forsberg. 1988. Polysaccharide Degradation by Rumen Microorganisms. In: Hobson, P. N. (Ed). Rumen Microbial Ecosystem., London: Elsevier Appl. Sci. p. 251-284.

Coen J. A. and B. A. Dehority. 1970. Degradation and utilization of hemicellulose from intact forages by pure cultures of rumen bacteria. Appl. Microbiol. 20:362-368.

Dehority B. A. and H. W. Scott. 1967. Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. J. Dairy Sci. 50: 1136-1141.

Flint H. J., C. A. McPherson and J. Martin. 1991. Expression of two xylanase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 cloned in pUC13. J. Gen. Microbiol. 137:123-129.

Fry S. C. 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Ann. Rev. Plant Physiol. 37:165-186.

Hungate R. E. 1966. The Rumen and Its Microbes. New York: Academic Press.

Joblin K. N. 1981. Isolation, enumeration, and maintenance of rumen anaerobic fungi in foll tubes. Appl. Environ. Microbiol. 42:1119-1122.

Lam T. B-T, K. Iiyama and B. A. Stone. 1990. Primary and secondary walls of grasses and other forage plants: Taxonomic and structural considerations. In: Akin D. E. Ljungdhal L. G., wilson J. R., and Harris P. J. (Ed). Microbial and Plant Opportunities to improve lignocellulose utilization by ruminants. New York: Elsevier Science Publishing. p.43-70.

Lowe S. E., M. K. Theodorou and A. P. Trinci. 1987a. Cellulase and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. Appl. Environ. Microbiol. 53:1216-1223.

Lowe S. E., G. W. Griffith, A. Milne, M. K. Theodorou and A. P. Trinci. 1987b. The life-cycle and growth kinetics of an anaerobic rumen fungus. J. Gen. Microbiol. 133:1815-1827.

Matsui H., K. Ushida and Y. Kojima. 1992. Fiber digesting extracellular enzyme profiles of fungal isolates *Neocallimastix* sp. N1 and *Piromyces* sp. P1 grown on five different carbohydrate. Anim. Feed Sci. Technol. 63:809-813.

Matsui H., K. Ushida, K. Miyazaki and Y. Kojima. 1988. Use of ratio of digested xylan to digested cellulose (X/C) as an index of fiver digestion in plant cell-wall material by ruminal microoganisms. Anim. Feed Sci. Technol. 71:207-215.

Morris E. J. and J. S. D. Bacon. 1977. The fate of acetyl groups and sugar components during the digestion of grass cell walls in sheep. J. Agric. Sci. 89:327-340.

Stewart J. C., A. Lester, B. Milburn and J. B. Parry. 1983. Xylanase and cellulase production by *Aspergillus fumigatus fresenius*. Biotechnol. Lett. 5:543-548.

Teunissen M. J., G. V. M. De Kort H. J. M. Op Den Camp and G. D. Vogels. 1993. Production of cellulolytic and xylanolytic enzymes during growth of anaerobic fungi from ruminant and nonruminant herbivores on different substrates. Appl. Biochem. Biotech. 39/40:177-189.

Ushida, K., C. Kayouli, S. DeSmet and J. P. Jouany. 1990. Effect of defaunation on protein and fibre digestion in sheep fed on ammonia-treated straw-based diets with of without maize. Br. J. Nutr. 64:765-775.

Van Soest, P. J. 1983. Nutritional Ecology of the Ruminant. Corvaillis, Oregon, USA: O &B Books, Inc.

Williams, A. G. and C. G. Orpin. 1987. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi grown on a range of carbohydrate substrates. Can. J. Microbiol. 33:418-426.